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Banana Ripening: Implications of Changes in Glycolytic Intermediate Concentrations, Glycolytic and Gluconeogenic Carbon Flux, and Fructose 2,6-Bisphosphate Concentration[1]

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ABSTRACT

In ripening banana (*Musa* sp. [AAA group, Cavendish subgroup] cv Valery) fruit, the concentration of glycolytic intermediates increased in response to the rapid conversion of starch to sugars and CO_2. Glucose 6-phosphate (G-6-P), fructose 6-phosphate (Fru 6-P), and pyruvate (Pyr) levels changed in synchrony, increasing to a maximum one day past the peak in ethylene synthesis and declining rapidly thereafter. Fructose 1,6-bisphosphate (Fru 1,6-P_2) and phospho*enol*pyruvate (PEP) levels underwent changes dissimilar to those of G 6-P, Fru 6-P, and Pyr, indicating that carbon was regulated at the PEP/Pyr and Fru 6-P/Fru 1,6-P_2 interconversion sites. During the climacteric respiratory rise, gluconeogenic carbon flux increased 50- to 100-fold while glycolytic carbon flux increased only 4- to 5-fold. After the climacteric peak in CO_2 production, gluconeogenic carbon flux dropped dramatically while glycolytic carbon flux remained elevated. The steady-state fructose 2,6-bisphosphate (Fru 2,6-P_2) concentration decreased to ½ that of preclimacteric fruit during the period coinciding with the rapid increase in gluconeogenesis. Fru 2,6-P_2 concentration increased thereafter as glycolytic carbon flux increased relative to gluconeogenic carbon flux. It appears likely that the initial increase in respiration in ripening banana fruit is due to the rapid influx of carbon into the cytosol as starch is degraded. As starch reserves are depleted and the levels of intermediates decline, the continued enhancement of respiration may, in part, be maintained by an increased steady-state Fru 2,6-P_2 concentration acting to promote glycolytic carbon flux at the step responsible for the interconversion of Fru 6-P and Fru 1, 6-P_2.

Detached fruits, unlike whole plants, provide an isolated system into which external carbon is not translocated and from which carbon losses only occur as volatiles which can almost completely be accounted for by respiratory CO_2. In a number of climacteric fruit, carbon is stored largely in the form of starch and as ripening proceeds is converted almost entirely to CO_2 and/or sugars. This is true for banana fruit in which starch constitutes approximately 20% of the fresh weight or about 85% of the dry weight of the pulp in mature unripe fruit and is almost completely converted to sugars and/or CO_2 as the fruit ripens (7, 30). Compared to the interconversion of starch to sugars and CO_2 during ripening, the synthesis or degradation of various other cellular constituents (*e.g.* organic acids, aromatic volatiles, lipids, protein, cellulose, lignin, and pectin) represents a minimal investment of carbohydrate (26) and provides a relatively low background of carbon interconversion. Thus, a reasonably accurate carbon balance sheet can be developed for ripening banana fruit in which the respiratory rate represents glycolytic carbon flux and the rate of sugar accumulation represents gluconeogenic carbon flux.

Starch is generally thought to be hydrolyzed within the amyloplast to triose phosphates (13, 21) which readily diffuse through the amyloplast envelope into the cytosol (13, 22). While available information concerning carbon movement across the envelope of intact amyloplasts and other plastids generally supports this view (13, 21, 22, 25), preferential transport of G-1-P[3] relative to other phosphorylated glycolytic intermediates across amyloplast envelopes and subsequent incorporation into starch has been demonstrated in developing wheat grain (17, 37). Additionally, significant transport of hexoses into chloroplasts takes place in spinach (29). No evidence, however, presently exists to suggest that hexose transport takes place in amyloplasts.

Within the cytosol, triose or hexose phosphates would be expected to predominantly enter into the Embden-Meyerhof-Parnas pathway to be utilized for the synthesis of sucrose and other sugars (gluconeogenesis) or CO_2 (glycolysis). During ripening, however, both the rate of CO_2 production and the sugar concentration increase. Thus, regardless of the point of entry of carbohydrate into the Embden-Meyerhof-Parnas pathway, carbon is simultaneously being shunted in both gluconeogenic (toward sucrose synthesis) and glycolytic (toward CO_2 synthesis) directions.

The enzymatic interconversion of Fru 1,6-P_2 and Fru 6-P

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[3] Abbreviations: G-6-P, glucose 6-phosphate; Fru 6-P, fructose 6-phosphate; Fru 1,6-P_2, fructose 1,6-bisphosphate; Fru 2,6-P_2, fructose 2,6-bisphosphate; G 1-P, glucose 1-phosphate; Pyr, pyruvate; PEP, phospho-enolpyruvate; FBPase, fructose 1,6-bisphosphatase; ATP-PFK, ATP-dependent phosphofructokinase; NTP-PFK, nucleotide-dependent phosphofructokinase; PPi-PFK, pyrophosphate: fructose 6-phosphate phosphotransferase or pyrophosphate-dependent phosphofructokinase.

is thought to be the most probable site of glycolytic control during fruit ripening (3, 5, 10, 18, 28, 41) and may be responsible for regulation of this bidirectional movement of carbon. Three enzymes are known to participate in the interconversion of Fru 1,6-P_2 and Fru 6-P: FBPase, ATP-PFK (NTP-PFK) and PPi-PFK. Under physiological conditions, FBPase operates in the gluconeogenic direction and ATP-PFK operates in the glycolytic direction, PPi-PFK, however, can operate in either direction. Historically, when this step in the glycolytic pathway was established as a critical control point during fruit ripening, the latter enzyme, PPi-PFK, had not been identified (9). As a consequence, the regulation of carbon flow through the glycolytic pathway during ripening needed to be reexamined. Recent studies indicate that the regulation by enzymes is complex and apparently involves changes in the cytosolic steady-state levels of Fru 2,6-P_2 (4, 5, 12, 18, 24). Fru 2,6-P_2 inhibits FBPase while activating PPi-PFK in both glycolytic and gluconeogenic directions. PPi-PFK favors the interconversion of Fru 1,6-P_2 and Fru 6-P in the glycolytic direction (*i.e.* the reaction catalyzed has a K_{eq} of 2–4 in the direction of CO_2 synthesis) (32) and exists as interconvertible large and small forms which, relative to one another, exhibit high and low activities, respectively (40). A high concentration of Fru 2,6-P_2 stabilizes the large form of PPi-PFK against dissociation into the less active small form by PPi (40). Increases in Fru 2,6-P_2 levels in vivo are therefore thought to promote glycolysis relative to gluconeogenesis through the simultaneous enhancement of PPi-PFK and the inhibition of FBPase (8, 15).

In ripening banana fruit, the majority of carbon movement is through the Embden-Meyerhof-Parnas pathway (1), thus, carbon interconversion can be examined via levels of phosphorylated intermediates and end product (sugar and CO_2) production rates. If enzymatic regulation remains constant, then changes in the pool size of intermediates should parallel changes in carbon flux. Conversely, non-parallel changes between intermediates and carbon flux or between two successive intermediates in a given pathway would indicate an alteration in enzymatic regulation. We examined which of these possibilities were operative by following temporal changes in Fru 2,6-P_2, the concentration of glycolytic intermediates and the magnitude and direction of carbon flux during the ripening of banana fruit with the goal of identifying the means by which bidirectional flux was regulated.

MATERIALS AND METHODS

Chemicals

All auxiliary enzymes and chemicals for intermediate assays were purchased from Sigma Chemical Company. Derivatizing agents for sugar analysis were purchased from Pierce Chemical Company.

Plant Materials

Mature, green, preclimacteric banana (*Musa* sp. [AAA group, Cavendish Subgroup] cv Valery) fruit were purchased from a commercial warehouse before they had been stored and gassed with ethylene to induce ripening. Data reported are from two individual lots of bananas obtained 9 months apart. Fruit were incubated at 22°C and internal levels of CO_2 and ethylene were continually monitored (see below). Fruit were individually assayed for sucrose, glucose, fructose, starch, Fru 2,6-P_2, G-6-P, Fru 6-P, Fru 1,6-P_2, Pyr, and PEP (see below) upon reaching predetermined stages of ripeness based on their internal ethylene levels and the time of the peak in ethylene synthesis. As ethylene production increased to peak rates, fruit were assayed when internal ethylene levels were 10 to 20, 30 to 100, 300 to 1000, 2000 to 5000 and >5500 $nL \cdot L^{-1}$. The latter sampling range is near the peak in ethylene synthesis and was taken as d 0. Dates corresponding with the sampling ranges prior to d 0 were estimated using a control group of banana fruit. Subsequent sampling times were 0.33, 0.67, 1, 2, 4 and 7 d after the peak in ethylene synthesis. Fruit were considered to have reached the climacteric stage (*i.e.* undergoing rapid physiological changes associated with ripening) when the internal ethylene concentration rose above 30 $nL \cdot L^{-1}$. Data points for all analyses represent the average of six fruit from replicates composed of 12 uniformly sized fruit selected from two banana 'clusters.' Clusters were paired based on size, shape and degree of 'filling out' (*i.e.* degree of roundness or loss of angularity of the fruit).

Determination of Internal CO_2 and Ethylene Concentrations

Ethylene and CO_2 concentrations were determined using GC. Gas samples for internal ethylene and CO_2 concentration were obtained from glass tubes fitted with rubber serum stoppers and attached to the surface of the fruit using Dow-Corning 3140 RTV noncorrosive silicone rubber as previously described (4) with a method adapted from Banks (2). Rates of CO_2 synthesis were not measured directly, but were estimated by correlating CO_2 production rates with internal CO_2 concentrations at the different stages of ripening using five representative fruit (data not shown). Changes in the internal concentration closely paralleled changes in production rates for CO_2 until day one. Afterwards, the ratio of the internal concentration to production rates decreased about 25 percent.

Determination of Sugars and Starch

Sugar and starch levels were determined from single tissue samples of approximately 0.5 g taken from a position near the fruit middle. Tissue samples were excised using a stainless steel knife and immediately frozen in liquid N_2. Frozen tissue samples were weighed, after which they were homogenized using a Virtis 45 for 10 min at high speed in a 20-mL glass vial containing 10 mL of methanol with 1 mg xylitol $\cdot L^{-1}$ as an internal standard. Assays for the internal standard at various extraction steps indicated xylitol underwent no apparent loss in recovery. Homogenizer blades were rinsed into the vial using an additional 10 ml methanol. Methanol was used to extract sugars and denature proteins. The methanol was drawn off overnight using heat (45°C) and low pressure (approximately 300 Torr). The above extraction procedure was repeated on this concentrated extract using 10 mL distilled water. The homogenizer blades were again rinsed with an additional 10 mL water. The vials were stored overnight

at 4°C which permitted additional extraction and allowed cellular debris to settle. Aliquots of 50 μL were removed, placed into 150 μL GC vials and evaporated at 40°C under a stream of N_2 gas. One hundred μL of the derivatizing agent (a 1:1 mixture of dimethylformamide and *N,O*-bis[trimethylsilyl]trifluoroacetamide) were added to each vial. The vial was sealed and the soluble sugars in the sample were derivatized by placing the vial in a heating block (78°C) and incubating for 90 min. The derivatized product was analyzed by gas chromatography (Hewlett Packard 5890a with a 5 m × 0.5 mm i.d. SE-54 wide bore capillary column, 250°C injection port temperature [splitless 0 to 2 min, split after 2 min], 100°C initial column temperature with a 6°C·min−1 rise for 30 min, 250°C flame ionization detector temperature with hydrogen as a carrier gas). Sugar concentrations were determined according to a standard curve.

After removal of an aliquot for soluble sugar analysis, the samples were prepared for starch analysis. The starch in the sample was gelled by incubating the 20-mL vials in a boiling water bath for 2 h. The samples were homogenized briefly and a 1-mL aliquot removed and placed in a 2-mL reaction vial. The starch in the samples was digested by adding 1 mL of a solution containing 25 units amyloglucosidase and 25 mM citric acid-citrate buffer (pH 4.6) to the reaction vial and incubating in a water bath at 55°C for 1.5 h. Following enzymatic hydrolysis of the starch to glucose, the solution in the vial was again subjected to soluble sugar analysis as above. The difference in glucose concentrations before and after incubation with amyloglucosidase was considered to represent the amount of starch in the sample. Incubation for a greater length of time or with a higher concentration of amyloglucosidase did not result in the production of additional glucose. This methodology permitted analysis of starch and sugars on the same tissue sample and thereby avoided variation introduced spatially separate tissue samplings.

The readily interconvertible carbohydrate pool (*i.e.* 'total' carbohydrate) was determined by summing hexose equivalents of starch, soluble sugars and estimates of respired CO_2 up to the stage at which tissue samples were taken. Contributions due to protein turnover, organic acid synthesis, aromatic volatiles, etc. were considered to be insignificant based on previous work (26). Variation in the total carbohydrate was corrected for by expressing the amount of starch, sugar and CO_2 as a percent of the total carbohydrate in the fruit prior to ripening. For sugars, the first derivative of the curve describing sugar concentration over time is the rate of carbohydrate conversion to sugars expressed as percent of the total carbohydrate converted per hour and is considered a measure of gluconeogenic carbon flux. In order to estimate glycolytic carbon flux, the production rate of CO_2, expressed as a percent loss of the total carbohydrate, was measured.

Determination of Glycolytic Intermediate Concentrations

The concentration of glycolytic intermediates was determined from transverse tissue discs, approximately 0.8 cm thick, sliced from the center of the banana and placed in liquid N_2. Approximately 5 g of the pulp tissue from the frozen discs was subsequently used for the assay. Intermediates were extracted using ice cold $HClO_4$ and their concentrations determined spectrophotometrically using methods adapted from Bergemeyer (6) as described by Kerbel *et al.* (18). Recovery experiments were conducted by adding glycolytic intermediates to the extraction medium at roughly twice the concentration encountered in the fruit extracts. Recoveries for G-6-P, Fru 6-P, Fru 1,6-P_2, Pyr, and PEP were 92.6, 94.6, 96.5, 101.3, and 101.5%, respectively. Since no attempt was made to isolate plastids prior to extraction, metabolite concentrations represent those in both plastids and cytosol.

Fru 2,6-P_2 Determination

Tissue samples for Fru 2,6-P_2 determination were taken from the center of the pulp at the middle of the fruit adjacent to samples taken for sugar and starch analysis. Samples of approximately 0.5 g were excised using a stainless steel knife and immediately frozen in liquid nitrogen. Frozen samples were ground with acid-washed Ottawa sand and 4 mL of a buffered grinding medium (50 mM Tris-HCl, pH 9.0) using a mortar and pestle and extracted as previously described (15). Fru 2,6-P_2 concentration of the extract was measured using the method of Van Schaftingen *et al.* (38).

RESULTS

Changes in Internal Ethylene, CO_2, and Sugar Concentrations

The changes in the internal concentration of ethylene and CO_2 shown in Figure 1 were nearly identical to those found previously (4). Ethylene levels began to increase rapidly 4 to 5 h before CO_2 concentrations underwent a similar rise. The ethylene concentration, however, rose more rapidly than that of CO_2 and consistently peaked about 24 h earlier. CO_2 concentration remained elevated in the latter stages of ripening while the ethylene concentration declined markedly.

Pulp soluble sugar concentrations began to rise noticeably

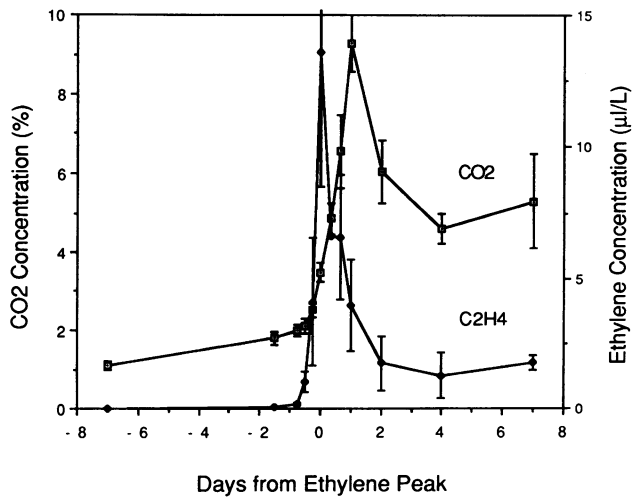

Figure 1. Internal concentration of ethylene (μL·L^{-1}) and CO_2 (%) in ripening banana fruit held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data points are the average of six fruit; bars represent 1 SD.

about 4 to 5 h after the onset of the respiratory climacteric and underwent their most rapid increase during the time period (d 0.67 to d 1) just prior to when the rate of CO_2 synthesis peaked (d 1) (Fig. 2). The concentration of sucrose increased 8 or more hours before any rise in fructose or glucose concentration could be detected. The sucrose concentration was greater than the combined concentrations of fructose and glucose throughout the ripening process and increased very rapidly in relation to the latter in the hours immediately following the peak in ethylene synthesis (Fig. 3). When concentrations of fructose and glucose did increase (approximately d 0.67 or d 1), they increased simultaneously and maintained a 1:1 ratio. Readily interconvertible 'total' carbohydrate did not change appreciably throughout ripening (Fig. 4). Starch degradation was accompanied by a concomitant increase in sugar concentration and the amount of CO_2 evolved. Changes in the internal concentration of CO_2 were closely paralleled by changes in its synthesis up to d 1 (data not shown). After d 1, the CO_2 production rate increased relative to the internal concentration, presumably due to differences in the permeability of the peel during ripening.

The rate of conversion of the total carbohydrate to sugars (*i.e.* gluconeogenic carbon flux) began to increase rapidly after d 0 (Fig. 5, A and B) and the extent of conversion approached 90% by d 7. The rate of conversion was most rapid from d 0.67 to d 1, having increased 50 to 100-fold since the preclimacteric minimum.

The rate of conversion of total carbohydrate to CO_2 (*i.e.* glycolytic carbon flux) began to increase rapidly in concert with gluconeogenic carbon flux (Fig. 5, A and B). With respect to preclimacteric values, glycolytic carbon flux increased only four- to five-fold by d 1. Moreover, glycolytic carbon flux increased more slowly than gluconeogenic carbon flux, peaking 4 to 8 h later (d 1). While the rate of gluconeogenic carbon movement decreased rapidly after d 1 to below preclimacteric rates by d 7, glycolytic carbon flux remained elevated relative to preclimacteric levels.

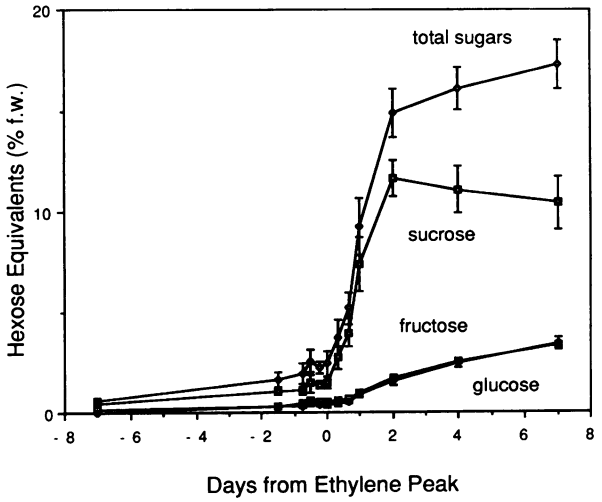

Figure 2. Hexose equivalents of fructose, glucose, sucrose and total sugars expressed as a percent of the fresh weight of ripening banana fruit held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data points are the average of six determinations; bars represent 1 SD.

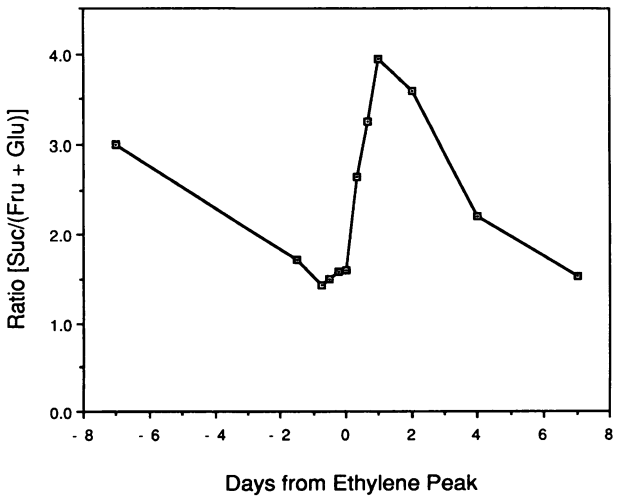

Figure 3. Ratio of hexose equivalents of sucrose to the sum of fructose and glucose in ripening banana fruit held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data points are the average of six fruit.

Changes in Glycolytic Intermediate Concentrations

Changes in the concentration of G 6-P, Fru 6-P (Fig. 6A) and Pyr (Fig. 6B) paralleled those of gluconeogenic carbon flux (compare with Fig. 5, A and B). Increases were generally synchronous with the onset of the respiratory climacteric and peak concentrations were detected at d 1, declining thereafter. Levels of G 6-P and Fru 6-P fell below those of the preclimacteric stages by d 4 and 7, respectively, whereas the concentration of Pyr did not.

Changes in the concentration of PEP (Fig. 6B) and Fru 1,6-

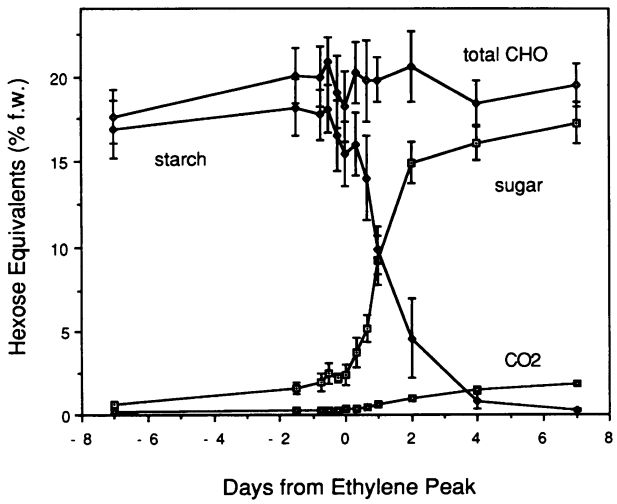

Figure 4. Hexose equivalents of starch, total sugars, respired CO_2, and their sum (*i.e.* total carbohydrates) of ripening banana fruit expressed as a percent of the fresh weight held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data points are the average of six determinations; bars represents 1 SD.

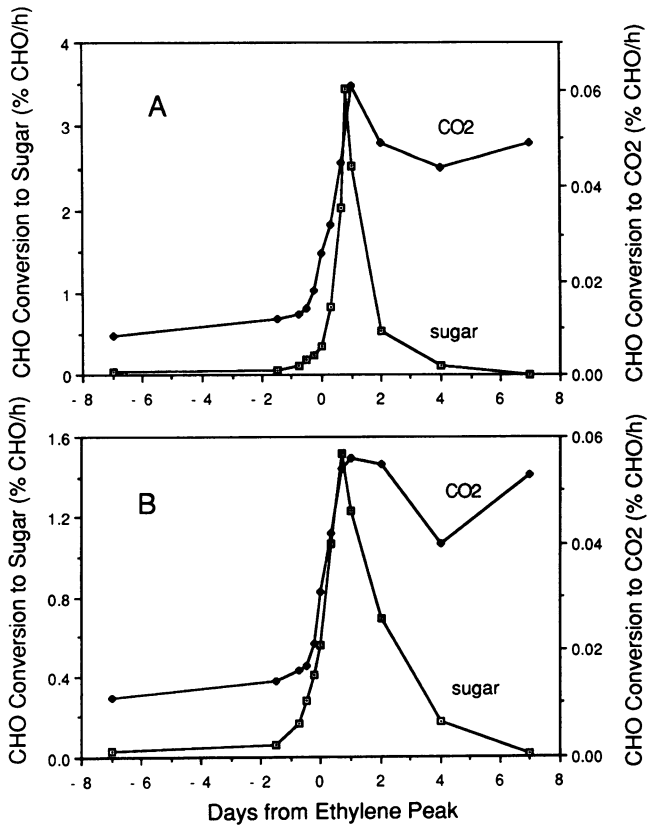

Figure 5. Superimposed curves characterizing the temporal relationship between glycolytic and gluconeogenic carbon flux expressed as the percent of the total carbohydrate converted to hexose equivalent of CO_2 and sugars per h, respectively, for ripening banana fruit held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data are the average of six fruit from lot 1 (A) and lot 2 (B).

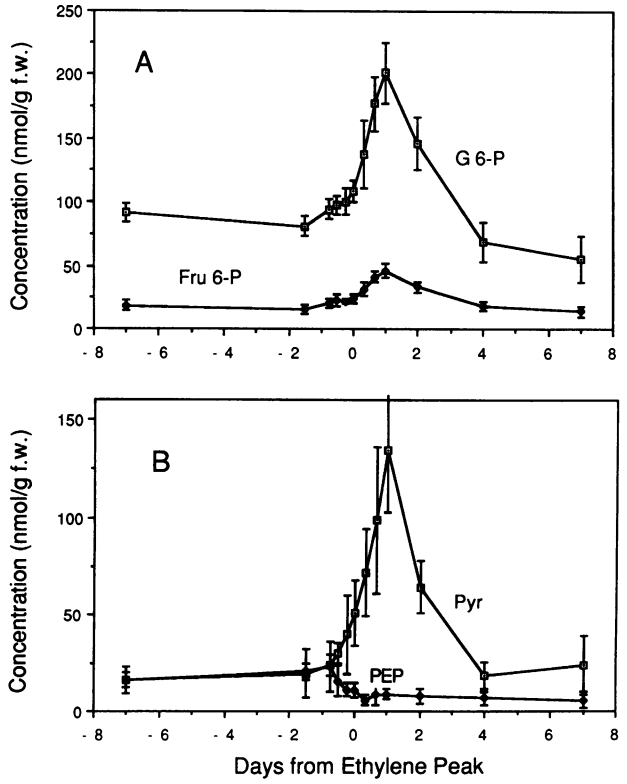

Figure 6. Concentration of (A) G-6-P and Fru 6-P and (B) Pyr and PEP in the pulp in ripening banana fruit held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data are the average of six fruit; bars represent 1 SD.

P_2 (Fig. 7A) did not parallel those of other intermediates examined. The PEP concentration was very similar to the Pyr concentration before the fruit entered the climacteric stage. After entry into this stage (*i.e.* when ethylene levels began to rise dramatically), PEP concentration declined until d 0 and remained low thereafter.

The Fru 1,6-P_2 concentration increased prior to that of Fru 6-P, but peaked later (Fig. 7A). As ripening continued, the Fru 1,6-P_2 concentration decreased, but not to the extent of the Fru 6-P concentration (*i.e.* not below preclimacteric levels). The ratio of Fru 1,6-P_2 to Fru 6-P began to increase rapidly on d 0 (Fig. 7B), which coincided with the peak in ethylene synthesis and the first significant increase in CO_2 production. The Fru 1,6-P_2/Fru 6-P ratio peaked at day 2 and remained elevated relative to values obtained prior to its initial increase.

Changes in Pulp Fru 2,6-P_2 Concentration

Fru 2,6-P_2 concentration changed after the fruit reached the climacteric stage in a manner consistent with previous Fru 2,6-P_2 determinations for ripening banana fruit (Fig. 8) (4). A transient peak in Fru 2,6-P_2 concentration occurred concurrent with the initial increase in ethylene, decreasing thereafter to its lowest levels from d 0.33 to d 1. Afterward, Fru 2,6-P_2 levels increased to about four times preclimacteric levels by d 7.

DISCUSSION

In the ripening banana fruit, it is assumed that carbon for sugar and CO_2 synthesis originates from the enzymic degradation of starch taking place within the amyloplast, which maintains its integrity throughout the ripening process. This is based on data from other plant tissues in which, during senescence, starch granules were degraded well before plastid integrity was lost and plastids remained intact even in the late stages of senescence (11, 14).

Glycolytic Intermediates and Carbon Flux

Cytosol and amyloplast metabolite pool levels were not separated in this study. However, it is assumed that the glycolytic intermediates in the cytosol comprise a major portion of the total intermediate pool as in maize endosperm amyloplasts (20). Therefore, unless otherwise noted, it is presumed in the following discussion that changes in the levels of glycolytic intermediates reported in this study are roughly representative of changes in glycolytic intermediates occurring in the cytosol.

Since respiration had removed only about 1% of the total carbohydrate by d 2 and since diversion of carbon into compounds other than CO_2 and sugars comprise a very low

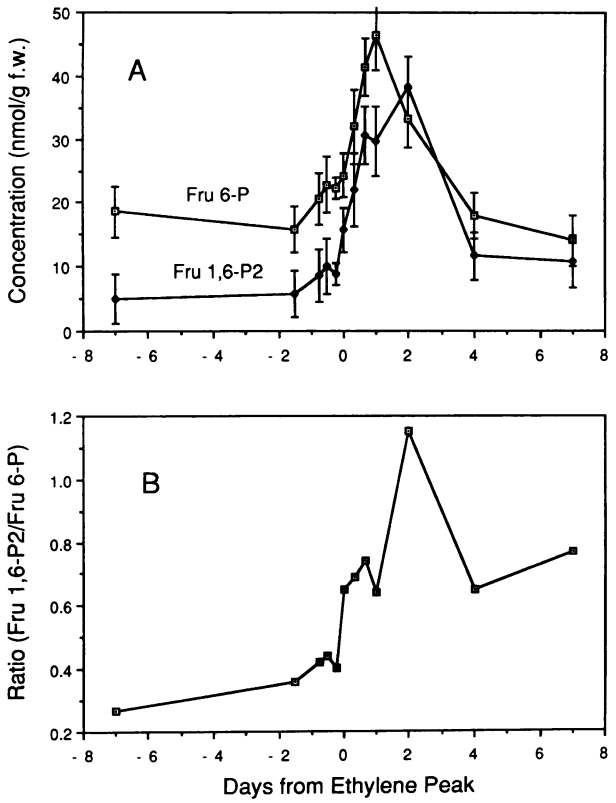

Figure 7. Concentration of (A) Fru 6-P and Fru 1,6-P_2 and (B) the Fru 1,6-P_2 to Fru 6-P ratio in the pulp of ripening banana fruit held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data are the average of 6 fruit; bars represent 1 SD.

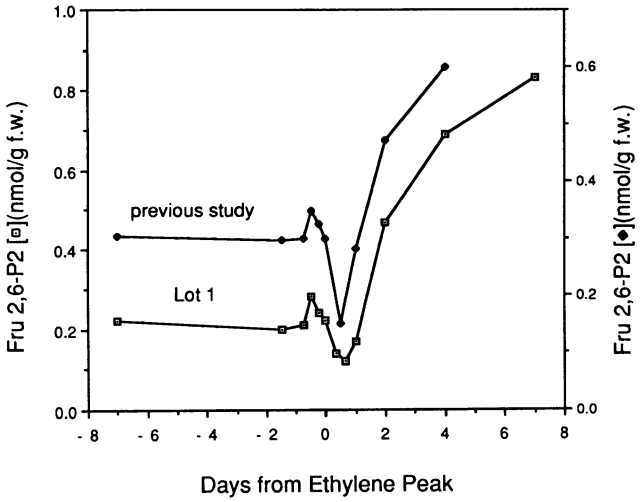

Figure 8. Concentration of Fru 2,6-P_2 in the pulp of ripening banana fruit from Lot 1 (□) and from a previously published study (4) (◆) held at 22 and 21°C, respectively from 7 d prior to 7 d after the peak in ethylene synthesis. Data are the average of six fruit (3 determinations per fruit in the previous study).

percent of the fresh weight (26), starch degradation within the amyloplast to phosphorylated glycolytic intermediates, carbon flux from the amyloplast as triose or hexose phosphates and gluconeogenic carbon flux through the Embden-Meyerhof-Parnas pathway all underwent similar changes in magnitude at least until d 2. If the enzymes regulating steps involved in this interconversion underwent no increase in activity, the concentration of phosphorylated intermediates situated between the feed-in and end points and/or in equilibrium with them should also have increased 50- to 100-fold by d 1. However, G-6-P and Fru 6-P (which are situated between starch and triose phosphates in the starch degradation pathway, between triose phosphates and sucrose in the Embden-Meyerhof-Parnas pathway of the cytosol and are in equilibrium with G-1-P in both cellular regions) underwent little more than a two-fold increase in concentration. This implies that the enzymic step(s) which limit the rate of starch degradation and/or those limiting sucrose synthesis in ripening banana fruit experienced marked increases in activity during the period of rapid starch conversion to sugar. However, since the levels of G-6-P and Fru 6-P represent the sum of those in the cytosol and plastids, it is not possible to determine which possibility is operative. The interconversion of G-6-P and Fru 6-P were probably not under any significant regulatory influence based on the close similarity between the temporal changes in G-6-P and Fru 6-P concentrations and the maintenance of approximately a 4:1 ratio, which are evidence that this interconversion is at equilibrium throughout ripening.

The dissimilarity of temporal changes in the concentration of PEP and Pyr indicates the regulation of the interconversion of PEP to Pyr changed as ripening proceeded (Fig. 6B). The marked decrease in PEP concentration relative to Pyr, suggests promotion of this interconversion began at the onset of the increase in ethylene synthesis (*i.e.* as the fruit was entering the climacteric stage). The drop in PEP levels may have also involved regulation of ATP-PFK, which is strongly inhibited by micromolar quantities of PEP (36). The decrease in PEP might therefore have resulted in increased glycolytic carbon flux through a stimulation of the Fru 6-P/Fru 1,6-P_2 interconversion step. Consistent with this possibility, ATP-PFK activity (4) and the ratio of Fru 1,6-P_2/Fru 6-P (discussed below) have been shown to increase more or less in coincidence with the decrease in PEP concentration.

The fourfold decrease in PEP concentration occurred simultaneously with a five- to six-fold increase in Pyr (Fig. 6B), indicating a promotion of the conversion of PEP to Pyr such that any PEP formed was immediately converted to Pyr. The magnitude and timing of the increase in Pyr concentration and glycolytic carbon flux were quite similar (four- to five-fold by d 1). The fact that Pyr serves as a substrate for carbohydrate oxidation to CO_2 in the mitochondria implies that the observed increase in Pyr concentration may be directly linked to the initial climacteric increase in respiration.

The dissimilarity of temporal changes in the Pyr concentration and glycolytic carbon flux after they peaked on d 1 indicates that conversion of carbon from pyruvate to CO_2 underwent a change in regulation. Since glycolytic carbon flux remained high despite the decline in Pyr availability as a substrate, it would be expected that an increase in the efficiency of Pyr catabolism occurred as the Pyr concentration dropped (d 2 to d 3). In fact, the oxidative decarboxylation of pyruvate increases several-fold in slices of climacteric banana

fruit fed Pyr (35). The activity of the decarboxylation step increases from essentially zero coincident with the respiratory rise. Compared to changes in respiration, however, the enzyme's activity rises more slowly and does not peak until about two days after the respiratory peak (*i.e.* what would correspond to approximately d 3 in this study).

The dissimilarity of changes in Fru 1,6-P_2 with glycolytic and gluconeogenic carbon flux and the Fru 6-P concentration during ripening (see Fig. 7, A and B, and compare with Fig. 5, A and B) is consistent with previous studies (10, 28, 41) showing that regulation of carbon flow occurs at this point in the pathway and that the degree of regulation changes as the ripening progresses. The increase in the Fru 1,6-P_2 concentration relative to Fru 6-P on d 0 (Fig. 7B) indicates that the movement of carbon was either facilitated slightly in the glycolytic direction or somewhat inhibited in the gluconeogenic direction. There is known to be an approximate 60% increase in ATP-PFK activity over preclimacteric levels on d 0 (4), which may account for the relative increase in Fru 1, 6-P_2.

The peak in the Fru 1,6-P_2 to Fru 6-P ratio on d 2 indicates a further increase in glycolytic promotion or gluconeogenic inhibition. All three enzymes catalyzing this interconversion may be involved: ATP-PFK activity remains elevated and exhibits a small peak on d 2; the ratio of PPi-PFK glycolytic activity of extract with added Fru 26-P_2 to that without added Fru 2,6-P_2 decreases on d 2, indicating a relative increase in the glycolytic capacity of the nonactivated enzyme extract by d 2; the level of Fru 2,6-P_2 increases 1.5- to 2-fold relative to preclimacteric levels, which may result in both the activation of PPi-PFK and the inactivation of FBPase. Although FBPase has a relatively low activity in banana compared to PPi-PFK (4), it has been implicated in the regulation of carbon partitioning. In spinach for instance, Fru 2,6-P_2 inhibition of FBPase has been proposed as a means of regulating gluconeogenic carbon flux (31). Generally, however, these glycolytic enzymes apparently undergo no extreme change in activity (4, 24, 41) that would account for the four- to fivefold increase in respiration or the 50- to 100-fold increase in gluconeogenic carbon flux.

After d 2, still elevated levels of Fru 1,6-P_2 relative to Fru 6-P indicate a continued promotion of glycolysis. The continually increasing Fru 2,6-P_2 concentration during this period may be partly responsible for maintenance of this ratio.

The importance of the Fru 6-P/Fru 1,6-P_2 interconversion step in the regulation of glycolysis is further supported by comparing preclimacteric and postclimacteric levels of the various phosphorylated intermediates. Only those in the glycolytic direction from this step were maintained at or above preclimacteric levels once starch degradation was completed. Carbon, therefore, appears to be preferentially shuttled in the glycolytic direction via changes in regulation at the interconversion of Fru 1,6-P_2 and Fru 6-P during the latter stages of ripening.

Carbohydrate Levels and Carbon Flux

In the past, considerable attention has been given to the increase in respiration during the ripening process for climacteric fruit, but the more significant event in the case of banana fruit is the conversion of starch to sugars. In the banana, between d 0 and d 2, the concentration of sugar increased from 10% to about 75% of the total carbohydrate. By contrast, only about 1 to 1.5% of the total carbohydrate was converted to CO_2 during the same time period. Thus, carbon movement within the glycolytic pathway was overwhelmingly in the direction of sugar production even as respiration increased to its maximal rate.

Sucrose, the primary sugar formed, increased in concentration rapidly relative to the summed concentrations of fructose and glucose (Fig. 3). The 1:1 ratio maintained between fructose and glucose throughout the ripening process (Fig. 2) suggests that fructose and glucose are produced via hydrolysis of sucrose. Hydrolysis of sucrose probably takes place in the vacuole, which actively accumulates sucrose (39) and is known to contain invertase (19). Thus, sucrose, synthesized from phosphorylated glycolytic intermediates in the Embden-Meyerhof-Parnas pathway, is apparently an intermediate in the synthesis of fructose and glucose from carbon stored originally as starch. If true, gluconeogenic carbon flux in the ripening banana fruit can also be taken to represent the rate of sucrose synthesis.

The recycling of carbohydrate from starch to sugars is an endergonic process (16) requiring phosphorylated nucleotides in the production of UDP-glucose. The rate of energy usage is one ATP equivalent per sucrose synthesized or 0.5 ATP equivalents per hexose equivalent converted to sucrose. That sugar production is dependent upon maintenance of glycolysis in banana fruit has been demonstrated with metabolic inhibitor studies. While the respiratory climacteric could proceed without other signs of ripening in banana fruit, ripening had an absolute dependence upon the elevated respiratory rate (23).

Calculation of the amount of carbon that must be oxidized to generate adequate energy required for gluconeogenesis is dependent upon the oxidative pathway used. If carbohydrate oxidation proceeded exclusively via the Embden-Meyerhof-Parnas pathway, the rate of nucleotide demand would require only about 1/72 of the hexose equivalents from starch hydrolysis to undergo complete oxidation. Therefore, gluconeogenic carbon flux could not exceed glycolytic by more than 72-fold. Due to differences in gas solubility, oxygen consumption exceeds CO_2 production by about 10 to 20% in the early stages of the respiratory climacteric (8, 26). Therefore, under conditions of tightly coupled oxidative phosphorylation and electron transport through the cytochrome *c* pathway, gluconeogenic carbon flux must remain below a theoretical maximum of about 80 to 90 times that of the glycolytic carbon flux. During the interval between d 0 to d 1, when increases in glycolytic and gluconeogenic carbon flux essentially paralleled one another and both reached their maximum, the rate of gluconeogenic carbon flux increased to a maximum of 60 to 70 times that in the glycolytic direction based upon best-fit curve analysis of the data. On the assay dates, however, the most that gluconeogenesis exceeded glycolysis was just over 40-fold. Therefore, the rate of glycolysis was more than sufficient to supply the energy necessary to form sucrose by a factor of 1.2 to 2.

The observed ratio between the glycolytic and gluconeo-

genic carbon flux rates precludes sole involvement of the pentose phosphate pathway, which would generate only 12 NADH per hexose equivalent oxidized. Each NADH from the cytosol can result in the production of 1.5 ATP (33) thus limiting ATP from respiration to approximately 18 per glucose. Each hexose equivalent oxidized could then only permit the synthesis of about 36 sucrose molecules, short of the 40 to 70 reported here. Estimated contributions of the pentose phosphate pathway range from 5 to 30% of the carbohydrate metabolized (1). Moreover, the pentose phosphate pathway is thought to be utilized to a decreasing extent as ripening proceeds (34).

If phosphorylated trioses are the form in which carbon exits the amyloplast during the ripening of banana fruit, then the ratio between glycolytic and gluconeogenic carbon flux rates would be indicative of carbon interconversion by PPi-PFK in the gluconeogenic direction. If FBPase were the sole enzyme used for gluconeogenic carbon flux in the cytosol, the energy requirement for sucrose production would be 2.5 ATP equivalents per sucrose synthesized and the number of hexose equivalents of sucrose formed per hexose equivalent of CO_2 produced could not exceed 16. Clearly, this was not the case, suggesting that if triose phosphates are the entry point into the Embden-Meyerhof-Parnas pathway then gluconeogenic carbon flux during ripening is dependent upon PPi-PFK, which, unlike FBPase, conserves energy by producing PPi when converting Fru 1,6-P_2 to Fru 6-P.

If a hexose phosphate (*i.e.* G 1-P, G 6-P or Fru 6-P) is the form that carbon exits the amyloplast, then the energetics of the interconversion are not changed; half an ATP equivalent is still needed per sucrose formed (16). The question of whether hexose or triose phosphates are the form of mobilized carbon that exits the amyloplast cannot, therefore, be answered on the basis of energetics of carbon interconversion in ripening banana fruit.

Fru 2,6-P_2 and Carbon Flux

For ripening banana, when the scales of glycolytic and gluconeogenic carbon flux are adjusted and the curves are superimposed such that the maximum flux in each direction is nearly equal (Fig. 7, A and B) one can visualize an interrelationship that appears to reflect real changes in ripening metabolism: glycolysis predominates over gluconeogenesis in the preclimacteric fruit; following entry of the fruit into the climacteric stage, gluconeogenesis rapidly increases relative to glycolysis; gluconeogenic carbon flux peaks slightly before glycolytic carbon flux by 4 to 8 h; and after both glycolytic and gluconeogenic carbon fluxes have peaked, glycolytic carbon flux remains relatively elevated.

Interestingly, the changes in the metabolic preference for either gluconeogenic or glycolytic carbon flux just described could also be used to describe the expected influence of the observed changes in Fru 2,6-P_2. In fact, if the scaled-down gluconeogenic carbon flux rates are subtracted from the glycolytic carbon flux rates, curves very similar to those describing changes in Fru 2,6-P_2 concentration result (compare Fig. 8 with Fig. 9, A and B). Fru 2,6-P_2 concentration, however, did not appear to be tightly associated with absolute promotion of either glycolysis or gluconeogenesis. In the ripening

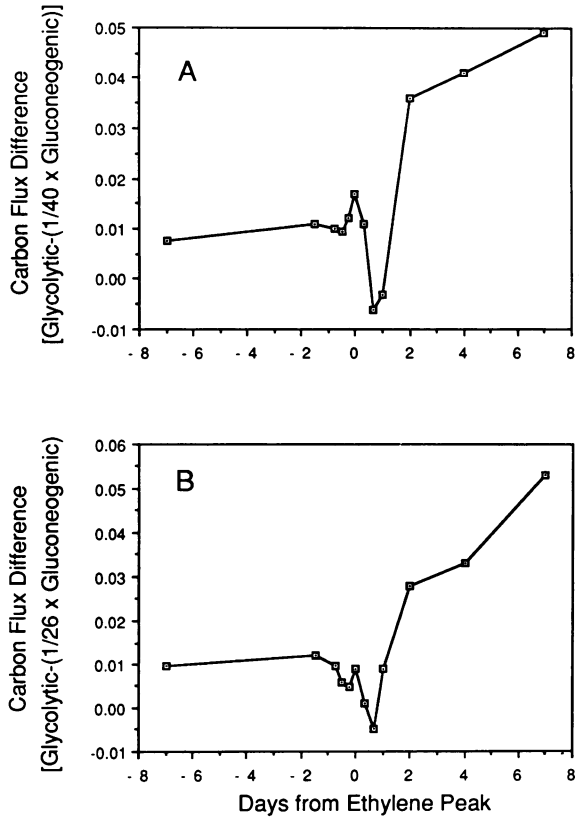

Figure 9. Difference between the glycolytic carbon flux and 1/40 (A) and 1/26 (B) times the gluconeogenic carbon flux in ripening banana fruit held at 22°C from 7 d prior to 7 d after the peak in ethylene synthesis. Data are the average of six fruit from lot 1 (A) and lot 2 (B).

banana, a low Fru 2,6-P_2 concentration coincided with the stage of greatest gluconeogenic activity (d 0.33 to d 1) and somewhat higher Fru 2,6-P_2 levels were associated with periods of relatively high glycolytic and low gluconeogenic carbon flux (d −7 to d 0 and d 2 to d 7). Steady-state levels of Fru 2,6-P_2 are, therefore, thought to exert their influence on the dynamic balance between gluconeogenesis and glycolysis such that an elevation of the basal Fru 2,6-P_2 exerts a relative preference for glycolysis under a given set of conditions without necessarily causing a net promotion of glycolysis over gluconeogenesis on a carbon for carbon basis.

The banana fruit, by virtue of the rapid and highly predictable manner with which it progresses through changes in carbon allocation during ripening, provides an excellent system to study the regulation of glycolytic and gluconeogenic carbon flux. Much of the means of fine control, however, needs to be elucidated. Clarification is needed, for example, regarding the feedback mechanisms which must undoubtedly be in place to meter carbon flow from the amyloplast in such a way that they match carbon flow into the mitochondria and vacuole while maintaining a comparatively steady availability of ATP and minute quantities of phosphorylated intermediates. Rapid fluctuations of the Fru 2,6-P_2 in pea (27) and banana tissue have been detected and may represent one means of fine control of the glycolytic pathway (RM Beaudry, unpublished data).

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